Chapter 11 – Biochemical Analysis of Gram Positive Species

Introduction and Background

Microbiologists will use a variety of agars to either identify the microbe using their respected biochemical properties that it displays once on that agar or to cultivate a specific microbe in an environment that it can only survive in. In this lab, we will be using two types of media to identify the biochemical properties of our microbes. These two types of media are selective media and differential media.

Selective media is intended to inhibit the growth of some microbes while permitting a select few to grow. Examples of selective media include, Mannitol Salt Agar (MSA) and eosin-methylene blue agar (EMB agar).

Differential media will allow for the growth of more than one microbe of interest but will be able to differentiate species (colonies) by specific biochemical properties that are unique to the microbe. This will typically contain a substrate and/or a pH indicator. Examples of differential media include DNase agar, Mannitol Salt Agar (MSA) and Triple Sugar Iron agar (TSI).

It is important to note that a particular media can be both selective and differential.

Below is description of the media that you will use in this lab:

Mannitol Salt Agar:

MSA is considered to be both selective and differential media. One always has to ask themselves the following questions: What is the agar selecting for? What are the ingredients present in MSA that select for it? MSA will select for halotolerant bacteria because it contains 7.5% NaCl in its agar.

The next questions one should ask are the following: What does MSA differentiate for and what in the agar allows one to see the change? The agar will allow for the differentiation of the microbes ability to ferment the carbohydrate **mannitol** or not. This is accomplished with the presence of the chemical indicator named **phenol red**. Below, in picture #1 is an uninoculated image of a MSA plate.

Picture #1: Uninoculated Mannitol Salt Agar plate.

This indicator will react with the acidic waste products produced if the microbe can ferment mannitol and will produce a **yellow color** (Positive for Mannitol Fermentation). If the microbe cannot ferment mannitol, then it will metabolize the proteins present in the agar and produce **alkaline** waste products. As a result the color of the agar will appear **pink/red** (Negative for Mannitol Fermentation). Below in picture #2 is a MSA plate that has been inoculated with a microbe that is mannitol fermenter (yellow) and a microbe that is not a mannitol fermenter (pink).

Picture #2: MSA plate that is inoculated with a mannitol fermenter (left) and a non-mannitol fermenter (right).

Pre-lab Questions:

1. List the ingredients of MSA.

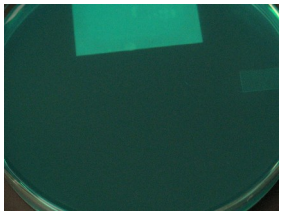
2. Which types of microbes can grow on MSA?

3. What is MSA differentiating for?

DNase agar:

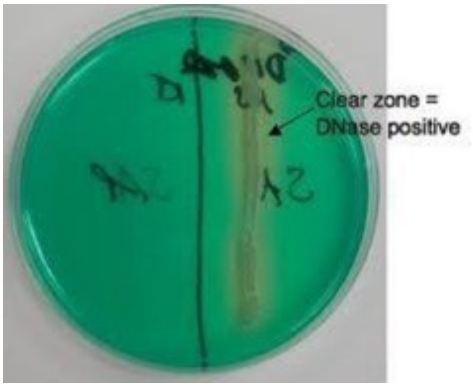
DNase agar is a differential media that is used to differentiate bacteria that have the genes to produce the DNase enzyme or not.

The agar has DNA, peptides, and methyl green dye in the agar that causes the agar to appear green. The DNase agar is pictured below in picture #3.



Picture 3: Uninoculated DNase agar.

Try and imagine that the dye is mixed in or "woven" with the methyl green dye. Bacteria that can secrete the enzyme DNase will be able to break down DNA and release the methyl green from the DNA. This will ultimately turn the color of the agar from **Green to Colorless**. The microbe is then said to be **DNase positive**. If the microbe does not have the genes to produce DNase, the agar will remain **green** and therefore, the microbe is said to be **DNase negative**. An example of this is seen below in Picture 4.



Picture 4: Inoculated DNase agar with DNase positive organism (Right) and DNase negative organism (Left).

Pre-lab Questions:

4. List the ingredients of DNase agar.

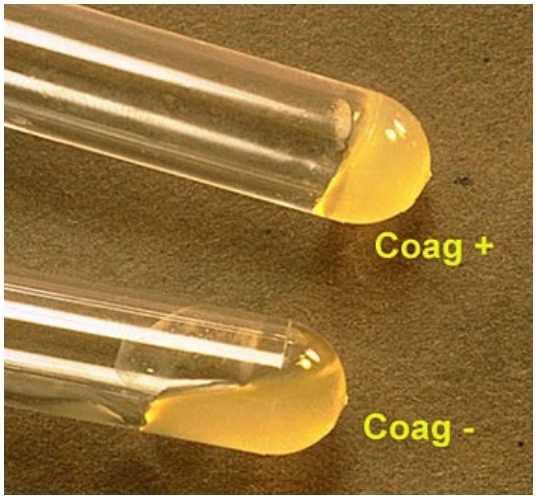
5. What do DNase negative microbes metabolize to survive?

Coagulase test:

The **coagulase test** allows for the differentiation of a microbes ability to convert fibrinogen to fibrin (clotting). If a microbe displays **clotting** then it is said to have to the genes to produce the enzyme **coagulase** and therefore the microbe is considered to be **coagulase positive**.

If the microbe does **not** have the genes to produce **coagulase** it will not be able to form a clot and therefore is considered to be **coagulase negative**.

The procedure to display coagulase activity in this lab will include a test tube of Brain Heart Infusion (BHI) broth and plasma. Below in picture 5 depicts a coagulase positive and negative reaction.



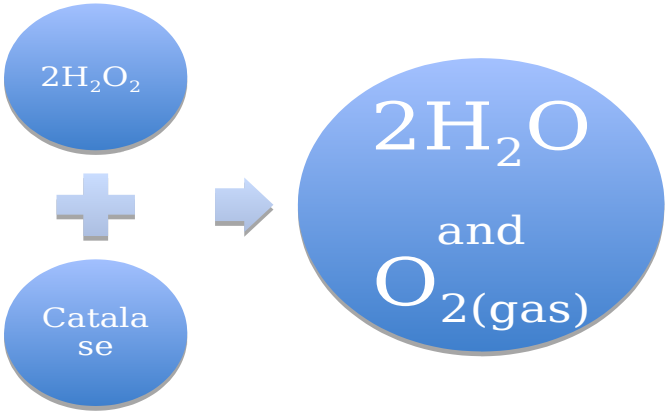
Picture 5: BHI broth and plasma inoculated with a coagulase positive bacteria (top) and a coagulase negative bacteria (bottom).

Pre-lab question:

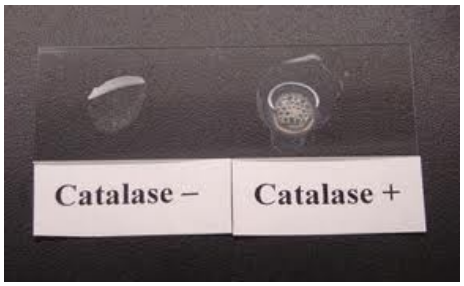
6. What is the advantage for the microbe to produce the enzyme coagulase?

Catalase Test:

The catalase test will help a microbiologist differentiate if a species has the genes to produce an enzyme called **catalase**. This enzyme has the ability to react with the toxic oxygen species like hydrogen peroxide (H_2O_2) and catalyze it into water (H_2O) and oxygen (O_2). The chemical reaction is described below:



If the enzyme catalase is present and hydrogen peroxide is added, then **oxygen gas or bubbles** will appear. The microbe is considered **catalase positive**. If the enzyme is not present, then **no gas** will be produced and the microbe is considered **catalase negative**. This can be observed in picture #6 below.



Picture #7: Glass slide with two different microbes and hydrogen peroxide. On the left, the microbes produced no bubbles, therefore they are catalase negative. On the right, the microbes produce bubble, therefore they are catalase positive.

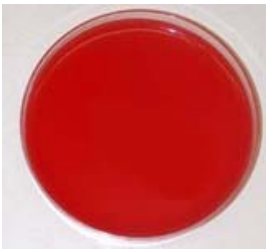
Pre-lab question:

7. What other enzyme can break down hydrogen peroxide and what are the products of that enzyme?

8. Why must a microbe break down hydrogen peroxide into water and oxygen gas?

Blood Agar:

Blood agar is considered an enriched differential media. It is enriched for culturing fastidious bacteria. Below in picture #8 is an example of uninoculated blood agar.



Picture #8: Uninoculated blood agar.

This agar has the ability to differentiate between microbes that can create exotoxins that will lyse the red blood cells in the agar, otherwise known as **hemolysis**. An example of these exotoxins includes the hemolysins. A microbiologist is also interested to see the degree on hemolysis caused by the microbe. The blood agar plate can differentiate this.

There are three types of hemolysis:

1. **Alpha** hemolysis is considered partial hemolysis. In addition, a greenish-grey discoloration of the blood will occur around the colonies.

2. **Beta** hemolysis is the complete lysis of red blood cells. This will change the color of the agar to turn completely colorless.

3. **Gamma** hemolysis is NO hemolysis present and will result in no color change in the agar.

Picture #9: Examples of alpha, beta, and gamma hemolysis on a blood agar plate.

Pre-lab Questions:

9. What are exotoxins?

10. What do hemolysins target?

11. What kind of bacteria produces hemolysins?

Bile esculin agar:

Bile esculin agar is both selective and differential. The bile salts will inhibit most Gram-positive organisms. The agar has bile salts, esculin, ferric ammonium citrate and other proteins. Below in picture #10 is an example of uninoculated Bile esculin agar in a slant.

Picture #10: Uninoculated Bile Esculin Agar slant.

The **esculin** is the substance that can or cannot be metabolized by microbes. This is the substance that gives the agar its differential properties. If the microbes have the genes to create the enzymes to

hydrolyze esculin, it is said to be **esculin positive**. The agar will turn **black**. The reason for this is that when esculin is metabolized, it becomes glucose and a substance known as esculetin. The esculetin will then react with ferric ammonium citrate to produce the black precipitate.

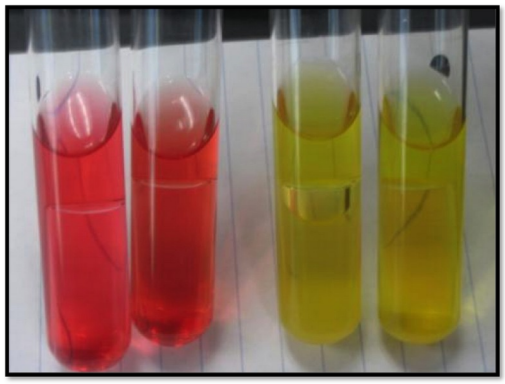
If the microbe does not have the genes to produce the enzymes to metabolize the esculin, then the agar remains **green** and is said to be **esculin negative**.

Picture #11: Inoculated Bile Esculin agar slants.

Phenol Red Mannitol Tubes:

The phenol red mannitol tubes are in a liquid state and will allow for the differentiation of the microbes ability to ferment the carbohydrate **mannitol** or not. This is accomplished with the presence of the chemical indicator named **phenol red**.

This indicator will react with the acidic waste products produced if the microbe can ferment mannitol and will produce a **yellow color** (Positive for Mannitol Fermentation). If the microbe cannot ferment mannitol, then it will metabolize the proteins present in the liquid and produce alkaline waste products. As a result the color of the liquid will appear **pink/red** (Negative for Mannitol Fermentation). Below in picture #12 are Phenol Red Mannitol Tubes that have been inoculated with a microbe that is mannitol fermenter (yellow) and a microbe that is not a mannitol fermenter (pink).



Picture #12: Phenol Red Mannitol Tubes that are inoculated with a mannitol fermenter (right) and a non-mannitol fermenter (left).

Materials:

- Wax Pencils (6 per Table)

- Disinfectant Bottles (2 per table)

- Test Tube Racks (2 per Table)

- Metal Inoculating Loops (6 per Table)

- Metal Inoculating Needles per table (6 per Table)

- Bunsen Burners and Hoses (2 of each per table)


- Boxes of Microscope Slides (1 per table)

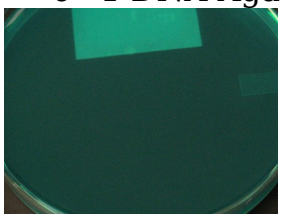
- "Waste" 500ml Beakers (1 per table)

- 6 Gram Positive Agars/Slant Set-ups (1 per table)
  - Each Set-up should have the following
    - 3 Bile Esculin Agar slants (for *Streptococcus* Species)

    - 3 Phenol red slants (for *Streptococcus* Species)
    - 1 Hydrogen Peroxide (Day 2)

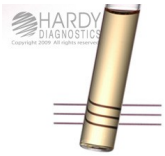
- 2 Blood Agar plates (for *Streptococcus* Species and *Staphylococcus* Species)

- 1 DNA Agar plates (*Staphylococcus* Species)



- 1 Mannitol Salt Agar plates (*Staphylococcus* Species)

- 2 Brain Heart Infusion tubes (*Staphylococcus* Species)



- 1 Blood plasma (Day 2)

Microorganisms needed:

- *Staphylococcus aureus*
- *Staphylococcus epidermidis*
- *Streptococcus pyogenes*
- *Streptococcus salivarius*
- *Streptococcus faecalis*

Procedures:

Catalase Test:

1. Take 5 glass slides and label each slide with one of the following microbes:
- *Staphylococcus aureus*
- *Staphylococcus epidermidis*
- *Streptococcus pyogenes*
- *Streptococcus salivarius*
- *Streptococcus faecalis*
2. Inoculate the glass slide aseptically with each respected microbe.
3. Add 1 drop of Hydrogen Peroxide on the microorganism.
4. Observe your results.
5. Take slides and dispose of them in the “Glass Waste” on the Instructors Bench.

Mannitol Salt Agar:

1. Take 1 MSA plate and dissect the plate in half with a marker. Label the plate with the following bacteria: *Staphylococcus aureus* and *Staphylococcus epidermidis*.

2. Inoculate the plate aseptically with the bacteria. DO NOT OVERLAP THE SPECIES.
3. Incubate your plates for 24 hours at 35 degrees Celsius.
4. Record the temperature of the incubator.
5. On day 2, observe your results and dispose of the agar plates in the Biohazards Waste.

DNase agar:

1. Take 1 DNase plate and dissect the plate in half with a marker. Label the plate with the following bacteria: *Staphylococcus aureus* and *Staphylococcus epidermidis*.
2. Inoculate the plate aseptically with the bacteria by streaking the plate as the capital letter "I". DO NOT OVERLAP THE SPECIES.
3. Incubate your plates for 24 hours at 35 degrees Celsius.
4. Record the temperature of the incubator.
5. On day 2, observe your results and dispose of the agar plates in the Biohazards Waste.

Coagulase Test:

1. Take 2 BHI broth tubes and label the tubes with the following bacteria: *Staphylococcus aureus* and *Staphylococcus epidermidis*.
2. Inoculate the tubes aseptically with the bacteria. DO NOT CROSS CONTAMINATE!
3. Incubate your plates for 24 hours at 35 degrees Celsius.
4. Record the temperature of the incubator.
5. On day 2, add 0.5ml of plasma to each BHI tube. Let it incubate for an hour.
6. Observe your results and return of the BHI tubes to your instructor's bench.

Blood agar:

1. Take 2 Blood agar plates and label them by dissecting one plate in half and the other plate in thirds.
- *Staphylococcus aureus*
- *Staphylococcus epidermidis*
- *Streptococcus pyogenes*
- *Streptococcus salivarius*
- *Streptococcus faecalis*

2. Inoculate the Blood agar aseptically with each respected microbe.
3. Incubate your plates for 24 hours at 35 degrees Celsius.

4. Record the temperature of the incubator.
5. On day 2, observe your results and dispose of the Blood agar plates in the Biohazards Waste.

Bile Esculin Slant Agar:

1. Take 3 Bile esculin agar (BEA) slants and label the GLASS with the following bacteria:
- *Streptococcus pyogenes*
- *Streptococcus salivarius*
- *Streptococcus faecalis*
2. Inoculate the slant of the BEA aseptically with each respected microbe. DO NOT STAB THE AGAR.
3. Incubate your plates for 24 hours at 35 degrees Celsius.
4. Record the temperature of the incubator.
5. On day 2, observe your results and return the BEA tubes to the instructor's bench.

Phenol Red Mannitol Tubes:

1. Take 3 Phenol Red Mannitol tubes and label the GLASS with the following bacteria:
- *Streptococcus pyogenes*
- *Streptococcus salivarius*
- *Streptococcus faecalis*
2. Inoculate the tubes aseptically with each respected microbe.
3. Incubate your plates for 24 hours at 35 degrees Celsius.
4. Record the temperature of the incubator.
5. On day 2, observe your results and return the Phenol Red Mannitol tubes to the instructor's bench.

REMINDER: YOU MUST DISINFECT YOUR BENCH AT THE END OF EACH CLASS AND CLEAN YOUR HANDS.

Results:

Catalase Test Results

Bacteria	Observations	Catalase +/-

Mannitol Salt Agar Results

Bacteria	Observations	Mannitol +/-

DNase Agar Results

Bacteria	Observations	Mannitol +/-

Coagulase Test Results

Bacteria	Observations	Coagulase +/-

Blood Agar Results

Bacteria	Observations	Hemolysis

Bile Salt Agar Results

Bacteria	Observations	Esculin +/-

Phenol Red Mannitol Tube Results

Bacteria	Observations	Mannitol +/-

Post-lab questions:

1. What is the purpose of this lab?

2. Why must you conduct all these different types of experiments?

3. What is the purpose of positive and negative controls?